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Simultaneous determination of the enantiomers of esmolol and its acid metabolite in human plasma by reversed phase liquid chromatography with solid-phase extraction

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Abstract

A stereoselective RP-high performance liquid chromatography (HPLC) assay to determine simultaneously the enantiomers of esmolol and its acid metabolite in human plasma was developed. The method involved a solid-phase extraction and a reversed-phase chromatographic separation with UV detection ($\lambda = 224$ nm) after chiral derivatization. 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) was employed as a pre-column chiral derivatization reagent. The assay was linear from 0.09 to 8.0 μg/ml for each enantiomer of esmolol and 0.07–8.0 μg/ml for each enantiomer of the acid metabolite. The absolute recoveries for all enantiomers were >73%. The intra- and inter-day variations were <15%. The validated method was applied to quantify the enantiomers of esmolol and its metabolite in human plasma for hydrolysis studies.

Keywords: Enantiomer separation; Esmolol

1. Introduction

Esmolol, methyl 3-{4-[2-hydroxy-3-(isopropylamino) propoxy]phenyl}propionate hydrochloride, is an ultra-short-acting β-adrenergic receptor antagonist used in the racemic form for the rapid control of heart rate in patients with atrial fibrillation or atrial flutter. Like most β-adrenergic blocking agents, (−)-*S*-esmolol exhibits blocking effects whereas (+)-*R*-esmolol is inactive [1]. In humans, esmolol has an in vivo elimination half-life of about 9 min. It is rapidly metabolized by hydrolysis of the ester linkage chiefly by esterases in blood to methanol and an acid metabolite, 3-{4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl} propionic acid (Fig. 1) [2–4]. In view of the importance and the potential enantioselectivity of this metabolic pathway, simultaneous determination of the enantiomers of esmolol and its acid metabolite seems to be more significant.

Enantioselective HPLC bioassays of the enantiomers are in general classified in two categories, indirect and direct method. In indirect methods, the enantiomers are separated through chiral derivatization to form diastereomers which are then separated on a non-chiral column. Direct methods require the use of chiral stationary phases or the addition of chiral additives to the mobile phases. Both methods have been applied to the resolution of the enantiomers of esmolol and other β-adrenergic blocking agents [5–10]. However, so far neither method was reported to quantify simultaneously the enantiomers of esmolol and its acid metabolite. In the present study, we developed and validated a sensitive, rapid and stereospecific method for simultaneous assay of esmolol and its acid metabolite enantiomers. The analytical assay involved solid-phase extraction of esmolol and its acid metabolite from human plasma followed by reaction at ambient temperature with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as a pre-column derivatization reagent. The diastereomers were then separated by reversed-phase high performance liquid chromatography (HPLC) with UV detection ($\lambda = 224$ nm). We also report a preliminary investigation

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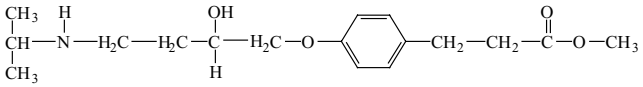
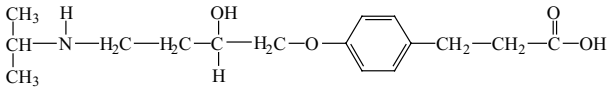


Esmolol



Acid metabolite

Fig. 1. Chemical structures of esmolol and its acid metabolite.

of the in vitro hydrolysis of esmolol enantiomers in human plasma.

2. Experimental

2.1. Chemicals

All solvents used were HPLC grade and all chemicals were analytical grade. Esmolol hydrochloride (purity >99.5%) was kindly provided by Zhan Wang Chemical Pharmaceutical Company (Huzhou, Zhejiang, China). 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and the internal standard (IS) (−)-*S*-propranolol were purchased from Sigma (St. Louis, MO, USA). 3-{4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl}propionic acid (purity >99.5%) was synthesized by our laboratory according to the method provided by Zhan Wang Chemical Pharmaceutical Company. Triethylamine (TEA) was obtained from Shanghai Chemical Reagent Plant (Shanghai, China). Drug-free human plasma was supplied by Ningbo Blood Center (Ningbo, Zhejiang, China).

2.2. Apparatus

The HPLC system consisted of a LC-10ATvp pump, a manual injector with a 20 μl fixed loop and a SPD-10Avp UV-Vis detector (Shimadzu, Japan). The diastereomeric derivatives from esmolol and the acid metabolite were separated on a 5 μm reversed-phase Aglient Zorbax C18 column (250 mm × 4.6 mm i.d.), equipped with a C18 guard column (10 mm × 5 mm i.d.) at ambient temperature. The mobile phase consisted of a mixture of acetonitrile/0.02 mol/l phosphate buffer (pH, 4.5) (55:45, v/v) and was delivered at a flow rate of 0.75 ml/min. Eluted peaks were detected at 224 nm. The chromatographic data were recorded and processed using a HS2000 Station Version 4.0 (Empire Science, Hangzhou, China).

2.3. Preparation of stock and standard solutions

Esmolol, the acid metabolite and (−)-*S*-propranolol were accurately weighted, transferred to volumetric flasks and dissolved in methanol to make individual stock solutions of 100 μg/ml of esmolol and the acid metabolite and 20 μg/ml of internal standard (−)-*S*-propranolol. The solutions were stored at 4 °C and were stable for at least 1 month. Stock solutions of esmolol and its acid metabolite were diluted to 10 and 50 μg/ml solutions with methanol, which were used for spiking human plasma.

2.4. Preparation of spiked human plasma samples

Appropriate amounts of the 10, 50 and 100 μg/ml standard solutions of esmolol and the acid metabolite were taken, and evaporated to dryness under a gentle stream of air. The residues were then reconstituted with drug-free human plasma to give final concentrations of 0.09, 0.2, 0.5, 1.0, 2.0, 4.0, 8.0 μg/ml for each enantiomer of esmolol and 0.07, 0.2, 0.5, 1.0, 2.0, 4.0, 8.0 μg/ml for each enantiomer of the acid metabolite.

2.5. Extraction and derivatization

Each plasma sample (0.5 ml) placed on ice was immediately mixed with 20 μl of the internal standard solution and 250 μl of 6% perchloric acid. After centrifugation at 4 °C (5000 × *g* for 10 min) to precipitate denatured proteins, the clear acid supernatant was transferred to a clean tube and mixed with 100 μl of 1.0 mol/l sodium hydroxide and 1.5 ml of 0.02 mol/l sodium phosphate buffer (pH 7.0). The mixture was applied to LC-18 SPE column (1.0 ml tube, Supelco Park, Bellefonte, PA, USA) preconditioned by sequential washing with acetonitrile (3.0 ml) and 0.02 mol/l sodiumn phosphate buffer (3.0 ml). The sample solution was allowed to run through by gravity and the cartridge was washed with 1.0 ml water. Excess of water was removed by leaving the cartridge in a vacuum system for about 30 min. The analytes were eluted from the cartridge with 3 ml of acetonitrile containing 1% glacial acetic acid. The eluent was evaporated to dryness under a gentle stream of air. 70 μl of GITC (1.02 mg/ml in acetonitrile) and 5 μl of TEA (1.25% in acetonitrile) were then added to the dried residue. The reaction was made at ambient temperature for 20 min. After chiral derivatization was completed, the reaction mixture was evaporated to dryness under a gentle stream of air and the residue was reconstituted with 100 μl of mobile phase. Finally, an aliquot of 20 μl of the resulting solution was injected into the HPLC system.

2.6. Hydrolysis study

Human plasmas (0.5 ml) spiked with different esmolol concentrations were incubated at 37 °C in the shaking water bath. Samples were taken from the shaking water bath at indicated time up to 8 h and then analyzed as described in Section 2.5.

3. Results

3.1. Selectivity

Fig. 2 shows the typical chromatograms of enantiomer derivatives of esmolol and its acid metabolite in blank human plasma and spiked samples. (−)-*S*-esmolol, (+)-*R*-esmolol, (−)-*S*-acid metabolite and (+)-*R*-acid metabolite were eluted at about 6.8, 7.7, 16.2 and 19.2 min. As demonstrated in Fig. 2, the present method was specific for determining each enantiomer of two analytes. The *S* and *R* enantiomers of each analyte were clearly well separated from the matrix components under the chromatographic conditions employed. In addition, the two enantiomers from each of two analytes were baseline-resolved from each other.

3.2. Linearity of calibration

Calibration curves were constructed by performing a regression linear analysis of the peak height ratios of the enantiomers to the internal standard versus the enantiomer concentrations. The calibration curves of each enantiomer were linear over the concentration ranges studied, i.e. (−)-*S*-esmolol, $y = 0.8529x + 0.0201$, $r = 0.999$; (+)-*R*-esmolol, $y = 0.7010x + 0.0304$, $r = 0.999$; (−)-*S*-acid metabolite, $y = 0.9342x + 0.0263$, $r = 0.998$; (+)-*R*-acid metabolite, $y = 0.9164x + 0.0282$, $r = 0.998$.

3.3. Precision and accuracy

The intra- and inter-day precision and accuracy were obtained by analyzing the spiked samples at three different concentrations of 0.2, 1.0, 4.0 μg/ml for the enantiomers of esmolol and its acid metabolite in five replicates within 1 day and on five consecutive days, respectively. The intra-day coefficient of variation and accuracy ranged from 3.5 to 9.5% and 93.6 to 110.5%, respectively. For the same concentration range, the inter-day coefficient of variation and accuracy ranged from 4.5 to 10.0% and 94.7 to 109.5%, respectively. The results are shown in Table 1.

3.4. Sensitivity

The limit of detection, defined as the lowest sample concentration which can be detected (signal-to-noise ratio, 3) was 0.008 μg/ml for each enantiomer of esmolol and 0.006 μg/ml for each enantiomer of its acid metabolite in human plasma, and the limit of quantification, defined as the lowest sample concentration which can be quantitatively determined with suitable precision and accuracy (signal-to-noise ratio, >10) was 0.09 μg/ml (R.S.D. = 12.6%, $n = 5$) for each enantiomer of esmolol and 0.07 μg/ml (R.S.D. = 13.2%, $n = 5$) for each enantiomer of esmolol acid metabolite.

3.5. Extraction recovery

Recoveries of esmolol and the acid metabolite enantiomers were assessed by comparing the peak heights of an extracted sample containing a known amount of enantiomers with the peak heights obtained from direct injections of a standard solution containing the same concentration of enantiomers. The results are shown in Table 2. The average method recoveries were 75.3% for (−)-*S*-esmolol, 74.5% for (+)-*R*-esmolol, 86.3% for (−)-*S*-acid metabolite and 85.4% for (+)-*R*-acid metabolite.

3.6. Hydrolysis study

The rate constant for the hydrolysis was calculated by linear regression analysis of log-transformed plasma concentrations of esmolol enantiomers versus time. The half-time ($t_{1/2}$) was calculated by equation

$$t_{1/2} = \ln\frac{2}{k},$$

where k is the hydrolysis rate constant.

Fig. 3 shows the mean plasma concentration of esmolol and the acidic metabolite enantiomers versus time, following addition of esmolol to the human plasma at 37 °C. The log-transformed plasma concentrations of esmolol enantiomers declined linearly against time until approximately

Table 1
Precision and accuracy data for esmolol and the acid metabolite enantiomers in plasma ($n = 5$) (see extraction and derivatization for experimental details)

Concentration spiked (μg/ml)	Concentration found $\bar{x} \pm s$			
	(−)-*S*-esmolol	(+)-*R*-esmolol	(−)-*S*-acid metabolite	(+)-*R*-acid metabolite
Intra-day ($n = 5$)				
0.2	0.219 ± 0.019	0.218 ± 0.017	0.221 ± 0.021	0.218 ± 0.018
1.0	1.057 ± 0.069	1.053 ± 0.065	0.936 ± 0.077	1.065 ± 0.084
4.0	4.164 ± 0.179	4.116 ± 0.144	4.136 ± 0.227	4.152 ± 0.237
Inter-day ($n = 5$)				
0.2	0.217 ± 0.018	0.215 ± 0.017	0.219 ± 0.022	0.218 ± 0.020
1.0	1.069 ± 0.068	1.048 ± 0.059	0.947 ± 0.077	1.047 ± 0.057
4.0	4.151 ± 0.241	4.192 ± 0.189	4.190 ± 0.268	4.201 ± 0.193

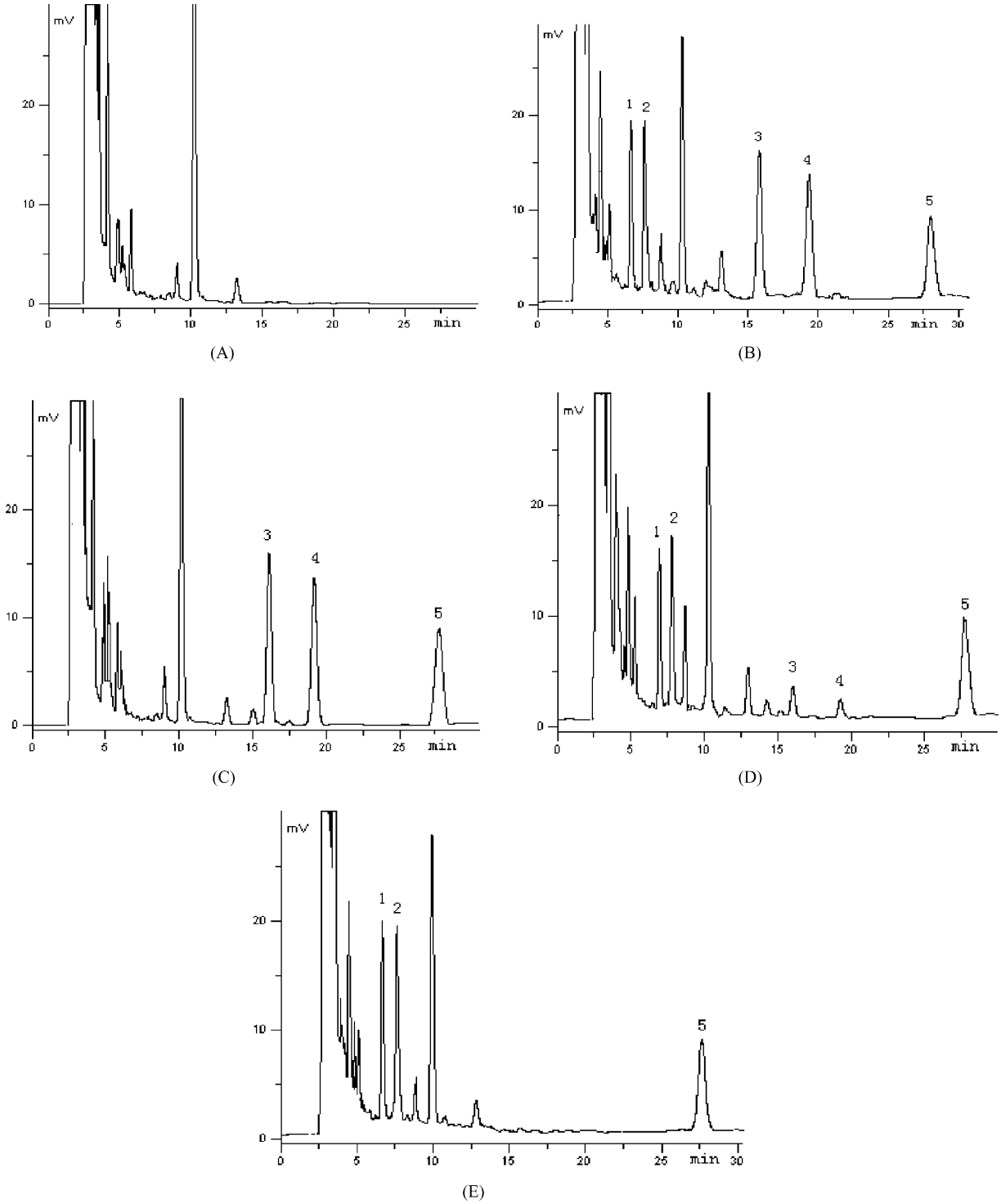


Fig. 2. Representative chromatograms of blank human plasma (A), blank plasma spiked with IS and 1.7 μg/ml of esmolol and the acidic metabolite (B), and plasma sample after hydrolysis for 0 h (C), 4.8 h (D) and 8 h (E): (1) (−)-*S*-acid metabolite; (2) (+)-*R*-acid metabolite; (3) (−)-*S*-esmolol; (4) (+)-*R*-esmolol; (5) (−)-*S*-propranolol.

90% of the added amount of esmolol was consumed. The hydrolysis of esmolol in human plasma resulted in the formation of an equivalent amount of the acid metabolite. Furthermore, the in vitro hydrolysis rate of (+)-*R*-esmolol in human plasma was faster than that of (−)-*S*-esmolol. The mean half-life of (−)-*S*-esmolol in human plasma was 2.48 h, and the corresponding half-life of (+)-*R*-esmolol was 1.82 h.

Table 2
Extraction recovery of esmolol and the acid metabolite enantiomers from plasma ($n = 3$) (see extraction and derivatization for experimental details)

Concentration spiked (μg/ml)	Recovery $\bar{x} \pm s$			
	(−)-*S*-esmolol	(+)-*R*-esmolol	(−)-*S*-acid metabolite	(+)-*R*-acid metabolite
0.2	76.9 ± 5.0	75.5 ± 4.4	88.5 ± 5.0	87.5 ± 5.3
1.0	74.1 ± 3.0	74.6 ± 3.3	84.2 ± 4.5	83.8 ± 4.6
4.0	75.0 ± 2.6	73.4 ± 1.9	86.1 ± 3.4	85.0 ± 3.7

4. Discussion

Due to the ester structure of esmolol, the in vitro stability of esmolol in human plasma was tested to ensure adequate handling of samples and optimal conditions for the assay. Plasma samples were placed on ice and 250 μl of 6% perchloric acid was added immediately. Perchloric acid inactivated the esterase and stopped the enzyme hydrolysis process, but at same time might result in the chemical decomposition of esmolol by acidifying the plasma samples. Our results showed that esmolol was stable for at least 5 h, which allowed enough time to handle the samples when samples were cooled down to 4 °C or kept on ice.

Since the zwitterionic nature of the acid metabolite which has both amino and carboxylic acid functions, it is difficult to extract simultaneously this metabolite with the basic parent drug, esmolol, from aqueous media using a simple liquid–liquid procedure. Previously reported studies [11,12] involved two independent procedures to determine esmolol and the acid metabolite in urine or blood. Esmolol was rapidly extracted from the assay matrix into the organic solvent and the acid metabolite remained in the aqueous phase. The determination of esmolol and the acid metabolite was then performed under the different chromatographic conditions. These methods are very laborious and time consuming. In the present study, esmolol and its acid metabolite were extracted simultaneously with solid-phase extraction using C_{18} reversed-phase cartridges. The absolute recovery was >83% for the acid metabolite enantiomers extracted from human plasma. Although lower recoveries were obtained for the esmolol enantiomers, it is quite acceptable (>73%) and reproducible (R.S.D. < 15%). In addition, the extraction efficiency of the acid metabolite using SPE method is highly dependent on the extraction condition, and a moderate rate of sample application and elution is desirable. Therefore, the sample was allowed to run though the SPE cartridge by gravity. The eluted solution is clear enough to remove interfering substance from human plasma and water that inactive chiral derivatization.

During the derivatization of esmolol and the acidic metabolite enantiomers, the amount of GITC as a pre-column derivative reagent, TEA as a catalyst and reaction time for the conversion of enantiomers to their corresponding diastereomers were optimized (Fig. 4). Results showed that the formation of the diastereomers was completed within 20 min after the addition of 70 μl GITC and 5 μl of TEA (1.25% in acetronitrile) at ambient temperature. Without the addition of TEA, the reaction yield of each enantiomer was <15% of the maximum in the presence of TEA, but the higher amount of TEA resulted in the reduction of the reaction yield and generated side products which caused interference. In order to avoid a kinetic resolution problem (i.e. GITC preferentially reacted with one enantiomer), a large excess of GITC was used [13,14]. It was founded that when the added molar amount of GITC was increased

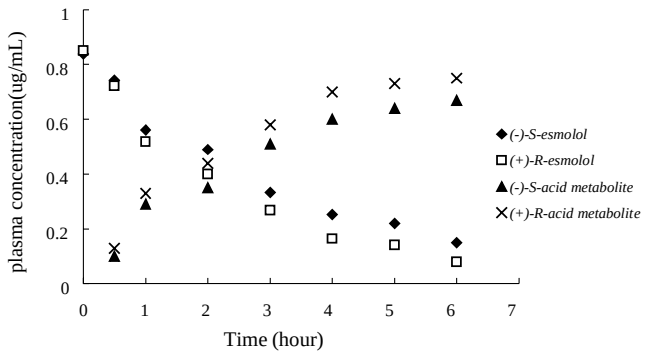


Fig. 3. The mean concentration of the enantiomers of esmolol and the acid metabolite vs. time in human plasma.

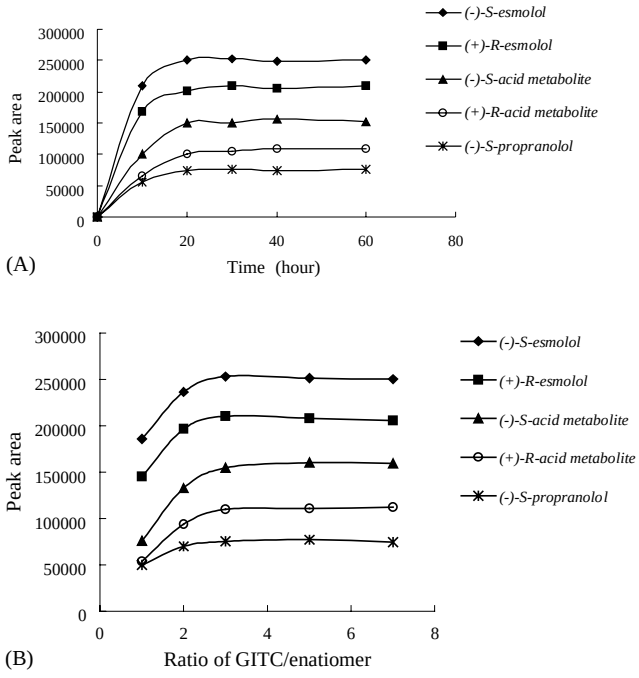


Fig. 4. Reaction time (A), ratio of GITC/enatiomer (B) and the c1onversion of enantiomers of esmolol and its acid metabolite and propranolol to the their corresponding diastereomers.

to three times that of the esmolol and the acid metabolite enantiomers, the reaction yield of each enantiomer reached to a maximum. In addition, the peak area ratios of (−)-*S*- to (+)-*R*-derivatives were calculated to be 1.00 ± 0.02, and no chiral inversion was observed in derivatization. Additional amounts of GITC did not spoil the chromatogram.

5. Conclusion

The enantiomers of esmolol and its metabolite have been successfully separated in human plasma by using solid-phase extraction and employing GITC as a pre-column derivatization reagent. The assay is rapid, accurate, sensitive and reproducible and allowed to monitor the concentration changes of each enantiomer of esmolol and the acid metabolite in the process of hydrolysis studies. Hydrolysis of two enantiomers of esmolol was stereoselective and in vitro hydrolysis rate of (+)-*R*-esmolol in human plasma was higher than that of (−)-*S*-esmolol.

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